PCR Detection and Microbiological Isolation of *Salmonella* spp. from Fresh Beef and Cantaloupes

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ABSTRACT: Species belonging to the genus *Salmonella* are an important cause of enteric fevers, gastroenteritis, and septicemia, and the pathogens are commonly transmitted through contaminated food. In this study, polymerase chain reaction (PCR) amplification of a 287-bp region of the *invA* gene was compared to a microbiological technique to determine the presence of *Salmonella* in retail beef and in cantaloupe rinse samples. Both methods showed the same level of sensitivity, detecting 1 CFU/25 g of meat after enrichment for 24 h at 42 °C. The presence of *Salmonella* was determined in 50 commercial top sirloin beef samples that were not artificially inoculated. Three samples were positive by the microbiological method, and these samples and an additional sample were positive by the PCR. Both methods were also used to test surface rinses of cantaloupes collected from 4 farms in Nayarit, Mexico. *Salmonella* was detected by the microbiological method in 9 of 20 samples (45%), whereas the pathogen was detected by the PCR in 11 samples (55%). This study demonstrates the utility of the PCR targeting the *invA* gene to determine the presence of *Salmonella* spp. in beef and cantaloupe samples.

Keywords: cantaloupe, detection, meat, PCR, *Salmonella*

Introduction

Salmonellosis caused by species in the genus *Salmonella* was described in 1984 as a "new and significant threat to the public health" by the World Health Organization (FAO 1984), and *Salmonella* has remained a major foodborne pathogen associated with different types of food. Gutiérrez and others (2000) reported the isolation of *Salmonella* spp. in Mexico in 51% of fast food samples, 23% of processed meat products (ham, chorizo, and bacon), 22% of ground food samples (beef, chicken, fish), 3% of milk products, and in 1% of both fresh and powdered eggs. Salmonellosis outbreaks in the United States linked to the consumption of cantaloupes implicated *Salmonella* serotypes Saphra and Poona as the causative agents from cantaloupes that originated from Mexico (Mohle and others 1999; CDC 2002). Analyses of fruits and vegetables imported to the United States in 1999 showed that of 1003 analyzed samples, 35 (3.5%) tested positive for *Salmonella* spp., and of these, 8 were cantaloupe samples (22.9%), indicating that cantaloupe was the 2nd most contaminated type of product after cilantro (FDA 2001). Currently in Mexico, the official procedure for detection of *Salmonella* spp. is a cultural method, and this procedure could take from 3 to 5 d for confirmation, which is a disadvantage when the results are needed promptly (SSA 1994; Peplow and others 1999). Molecular methods, such as the polymerase chain reaction (PCR), have shown high sensitivity and specificity for detecting target pathogens, including *Salmonella,* in different types of foods, and the time required to obtain results can be as short as 12 h (Ferretti and others 2001; Croci and others 2004). However, microbiological techniques are used as reference methods to demonstrate the efficacy and validity of new techniques (Fernandez 2000). The objective of this study was to compare the sensitivity of a PCR assay to a microbiological method and to evaluate the 2 methods for the detection of *Salmonella* spp. in naturally contaminated beef and cantaloupe rinse samples.

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Materials and Methods

Bacterial strain, growth conditions, and preparation of inoculum

S. Typhimurium ATCC 13311 was grown on trypticase soy agar (TSA) (Becton Dickinson Co., Sparks, Md., U.S.A.) at 37 °C for 24 h. Afterward, 10 mL of physiological saline solution (0.85%) were added to the plate to obtain a homogeneous suspension of bacteria. An aliquot of the suspension was diluted to a concentration of the nr 5 tube of the MacFarland scale (1.5×10^8 CFU/mL). Ten-fold serial dilutions were prepared in saline solution to give suspensions containing 10^0 to 10^4 CFU/mL.

Artificial inoculation of top sirloin meat samples

The top sirloin meat samples were obtained on the same date from supermarkets in Monterrey, Nuevo León, Mexico. Meat samples were placed into a cooler (at 4 °C) and transported to the laboratory. The samples were immediately separately inoculated with the previously mentioned dilutions as described subsequently. Three sterile plastic bags (Whirl-Pak®, Nasco, Modesto, Calif., U.S.A.) with the capacity of 500 mL were labeled for each dilution, and 25 g of top sirloin meat were weighed and placed in each plastic bag with 225 mL sterile buffered peptone water (BPW) (Becton Dickinson and Co.). An uninoculated control sample was

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included to ensure that the meat was not naturally contaminated with *Salmonella*. One milliliter of each bacterial dilution (10^0 to 10^4 CFU/mL) was added to the corresponding plastic bag, which was then mixed by hand for 2 min followed by incubation at 35 °C for 24 h. The enrichment and microbiological analyses were performed using tetrathionate (TT) broth (Becton Dickinson Co.) according to the method described in the Microbiology Laboratory Guidebook (FSIS-USDA 2004). The experiments were performed in triplicate.

DNA extraction from inoculated top sirloin meat samples

Three milliliters from the Tetrathionate broth enrichments were used to form cell pellets by centrifuging at 3000 rpm for 5 min. DNA extraction from the cell pellets was performed using the CTAB (cetyl trimethyl ammonium bromide) method; however, the use of polyvinylpyrrolidone and ß-mercaptoethanol was omitted (Doyle and Doyle 1987). The extracted DNA was stored at −20 °C.

PCR amplification conditions

Amplification of the target sequence was performed using a PCR Express thermal cycler (PCR Express; Thermo Hybaid, Middlesex, U.K.). The PCR mixture contained 25 pmoles of each of the primers targeting the *invA* gene (Rahn and others 1992), 200 μM of each of the 4 deoxynucleoside triphosphates (Bioline Inc., Randolph, Mass., U.S.A.), 1 mM $MgCl_2$, 1× Reaction Buffer (200 mM Tris-HCl pH 8, 500 mM KCl), 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis., U.S.A.), 100 ng of DNA template, and deionized water for a final volume of 25 μL. The reaction mixture was subjected to the following thermal cycling conditions: heat denaturation at 95 °C for 1 min, and then 35 cycles with heat denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30 s, and DNA extension at 72 °C for 30 s. After the last cycle, samples were maintained at 72 °C for 10 min to complete synthesis of all strands. The PCR products were subjected to gel electrophoresis (1.5% agarose; Promega), and then stained with ethidium bromide (0.5 μg/mL), visualized with a UV transilluminator (Spectroline Transilluminator, Model 7C-254R. Electronics Corp., Westbury, New York, U.S.A.), and photographed.

Detection of *Salmonella* spp. in samples of commercial bovine meat

Fifty different samples of bovine meat (New York Strip and Rib Eye) obtained from different supermarkets located in the metropolitan area of Monterrey, N. L. Mexico, were collected during the month of May in 2005 to determine if the meat was naturally contaminated with *Salmonella* spp. Handling of the samples and the microbiological and PCR methods were performed as described previously.

Detection of *Salmonella* spp. from rinses of cantaloupe surfaces

Cantaloupe melons were obtained at 4 fields in Nayarit, Mexico, on 4 different dates from February 25 to April 11 of 2005 (1 field per date). Each field was divided into 5 representative quadrants (I to V). Twenty-five melons were collected from each field, 5 melons per quadrant. The melons were randomly collected, and adhered particles of soil were not removed. Melon surface washings were done at the field. Each melon was placed in a sterile Whirl-Pak bag using gloves that were changed with each melon. Twenty-five milliliters of 0.1% sterile buffered peptone water were added to each plastic bag, and the fruit was washed thoroughly by shaking and mixing the bag by hand for at least 2 min. The washings from the 5 melons obtained from the same quadrant were combined into a single sterile glass bottle, and then placed into a cooler (at 4 °C) and transported to the laboratory. Processing of the samples was performed within 24 h of obtaining the rinses. At the laboratory, each sample was mixed by shaking, and 25 mL were removed and added to 225 mL of BPW to continue with the microbiological method and the PCR assay as described previously.

Results and Discussion

Isolation of *Salmonella* spp. from artificially inoculated meat samples and detection by the PCR

The PCR assay and the microbiological method showed an equal level of sensitivity with a limit of detection for *S.* Typhimurium by both methods of 1 CFU/25 g of meat (initial inoculum level) (data not shown). The primer pair targeting the *invA* gene, designed by Rahn and others (1992), was tested using a collection of 630 *Salmonella* strains and showed 99.4% specificity and no amplification of DNA from non-*Salmonella* strains. Moreover, the adequacy of *invA* as a target gene in PCR assays was tested by Daum and others (2002) in a fluorogenic TaqMan PCR assay to confirm the presence of *Salmonella* directly from chicken in less than 3 h. Although in the current study a single serovar was tested using this assay, we have also amplified the PCR product of the *invA* gene in serovar *S.* Enteritidis using the same primer pair in assays for the detection of *Salmonella* in other types of food samples (unpublished data). Thus, the PCR assay targeting the *invA* gene can potentially be used to detect *Salmonella* in raw beef samples as an alternative to the cultural method. However, enrichment is a necessary step if fresh products are tested by either the microbiological method or the PCR, since it is possible that a low level of the pathogen of interest may be present. Growth of the target organism, however, could be inhibited somewhat by the accompanying flora, which might give a negative result by the cultural method, or the sensitivity of the PCR assay could be decreased. As such, it is important to select a suitable enrichment medium to inhibit the background flora and the appropriate enrichment time because PCR sensitivity tends to increase with increases in enrichment times (Guo and others 2000).

Microbiological assay and PCR of commercial meat samples

The data in Table 1 show results of 4 samples, which were positive by the PCR assay and the results of the same samples tested using the cultural method for the detection of *Salmonella* spp. The other 46 meat samples analyzed were negative using both methods. Three samples out of 50 (6%) were detected as positives by the microbiological method, while the PCR assay detected an additional sample (8%). Several reasons could explain why a sample was detected as positive by the PCR (sample nr 12) and not detected as positive by the microbiological method. It is possible that the cells in the meat sample were injured due to the use of sanitation products or due to stressful storage conditions, which can damage or even kill the *Salmonella*. This in turn would affect the ability to detect the pathogen using the cultural method, since this method is dependent on growth of the cells. Figure 1 shows the

Table 1—Meat samples positive for *Salmonella* spp., by both the cultural and PCR methods.

Sample nr	Cultural method	PCR assay
3	+	+
12	−	+
33	+	+
41	+	+

PCR products obtained following amplification of the positive meat samples. Sample nr 12 (lane 3) shows a weak band. Thus, it is possible that there was less PCR product from this sample because there were a lower number of cells after enrichment compared to the other samples. This was likely the reason why the pathogen was not detected by plating. Although, the sensitivity of both the microbiological and PCR methods was the same (1 CFU/25 g) when meat samples were inoculated with nonstressed cells, in naturally contaminated samples, the cells may be stressed affecting their ability to grow as rapidly as nonstressed cells in the enrichment medium.

Cultural and PCR assays using cantaloupe rinse samples

The data in Table 2 show that in the 1st field, 4 positive samples were detected (quadrants I to III and V) by the microbiological method, and there were 4 positives from the same 4 quadrants by the PCR. However, for quadrants III and IV, the results of both methods differed. In the 2nd field, the results of both methods were the same, with positive results from samples in all 5 quadrants. In the 3rd and 4th fields, all results using the microbiological assay were negative, while using the PCR, results were positive from samples from the 3rd field from quadrants II and III and were negative from the 4th field from all 5 quadrants. With the exception of samples from quadrant III and IV from the 1st field where results differed, results of the PCR assay agreed with those of the cultural method, and in some cases the PCR assay was more sensitive for detection of *Salmonella* (quadrants II and III of the 3rd sampling). In summary, *Salmonella* spp. were detected by the microbiological method in 9 of 20 samples (45%), whereas the pathogen was detected by the PCR in 11 samples (55%). These results are partially in agreement with those of Espinoza-Medina and others (2006) who found that by the PCR method, 25.7% of samples from in-field cantaloupes were positive for *Salmonella*, whereas no positive samples were detected by the standard method.

The detection of this pathogen by the PCR was done from an enrichment culture; therefore, in addition to growth of *Salmonella*, the microflora from the melon samples also grew. Thus, the PCR was sensitive and specific, since *Salmonella* was detected in the presence of other microorganisms found in the melon production environment. Previous microbiological studies conducted in the Lagunera Region of Mexico (Froto and others 2004) found the presence of bacteria that belonged to the Enterobacteriaceae family and other microorganisms in the cuticle of melons, as well as plant pathogens such as *Fusarium* spp., *Verticillium* spp., and *Rhizoctonia solani*, and saprophytes such as *Aspergillus* spp., *Rhizopus* spp., and *Penicillium* spp. Also, human pathogens, including *Clostridium botulinum*, *Listeria monocytogenes*, *Vibrio cholerae*, *Brucella melitensis*, *Salmonella* Typhi, *Salmonella* Paratyphi, hepatitis A virus, *Escherichia coli*, and *Shigella dysenteriae*, were found. Most of these pathogens could be found in the soil coming from bovine and avian manure and from human feces (Froto and others 2004). The absence of the *Salmonella invA* sequence in other invasive bacteria such as *Yersinia* spp., *Shigella* spp., and enteroinvasive *E. coli*, which also have the capacity to invade epithelial cells, demonstrates the particular specificity and utility of this primer pair for detection of *Salmonella* spp. (Galán and Curtiss 1991). The *invA* gene has been used as the target in PCR assays mainly for detecting *Salmonella* in poultry, meats, and dairy products, and in vegetables and fruits (Guo and others 2000). The PCR results of the current study shown in Figure 2 indicate that the primers could be used for detection of *Salmonella* from cantaloupe surface washings and potentially in other types of fruits and vegetable samples, as well.

The differences in results from field to field may have been due to differences in levels of *Salmonella* contamination. This may have been influenced in part by changes in the environment, including loss of specific nutrients and fluctuations in humidity, temperature, and ultraviolet light, all of which could damage bacterial cells and

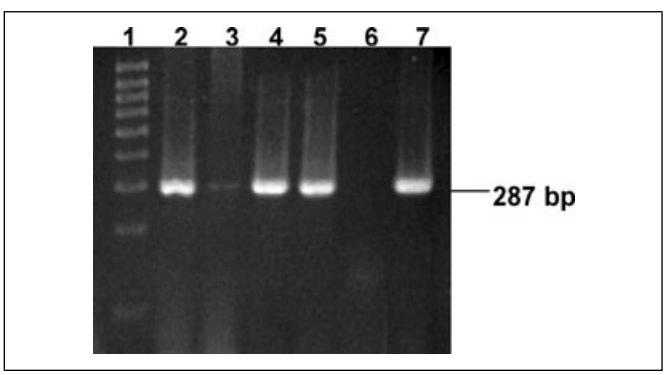

Figure 1—PCR products from the *Salmonella invA* gene in beef samples positive for *Salmonella* spp. Lane 1, molecular weight markers, 100-bp ladder (BIOLINE); lanes 2 to 5, samples 3, 12, 33, and 41, respectively; lane 6, negative control; and lane 7, positive control—*S.* Typhimurium.

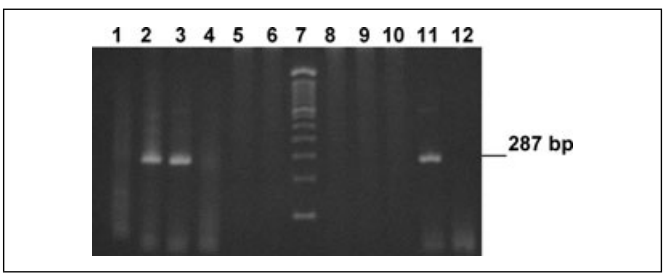

Figure 2—Detection of *Salmonella* spp., by the PCR from cantaloupe surface washings. Lanes 1 to 4, quadrants I to IV (3rd field); lanes 5 to 6 and 8 to 10, quadrants I to V (4th field); lane 7, molecular weight marker, 100-bp ladder (BIOLINE); lane 11, positive control—*S.* Typhimurium; lane 12, negative control.

Table 2—Positive results for *Salmonella* spp. from cantaloupe surface washings using the cultural method and the PCR assay.

	Assay results							
	First field		Second field		Third field		Fourth field	
Quadrant	Microbiological	PCR	Microbio logical	PCR	Microbiological	PCR	Microbiological	PCR
I	+	+	+	+	−	−	−	−
II	+	+	+	+	−	+	−	−
III	+	−	+	+	−	+	−	−
IV	−	+	+	+	−	−	−	−
V	+	+	+	+	−	−	−	−

combined with the low probability that human pathogens develop stress resistance (Dickinson 1986; O'Brien and Lindow 1988). Furthermore, each field was sampled only once during the 1.5 mo period, and Good Agricultural Practices (GAP) were not applied in the first 3 fields. For the 4th field, in which no positive results were obtained, the cantaloupes were obtained from a field in which GAP were applied. These included the use of plastic mulch, fertirrigation, water without evidence of microbiological contamination, portable restrooms in the fields, training of field workers in GAP, use of authorized pesticides, designation of areas where workers may eat and take breaks, and availability of potable water for workers, which according to HACCP (Hazard Analysis and Critical Control Point) plans are basic tools for reducing physical, chemical, and microbiological hazards in agricultural production.

Conclusions

Although meat is usually not consumed raw, there is risk of *Salmonella* infection if the meat is improperly cooked, and there is also the possibility of cross contamination of *Salmonella* with foods that are consumed raw. Produce may become contaminated in the field through the use of contaminated irrigation water or manure or also from animals or inadequate worker hygiene. Therefore, the ability to rapidly detect *Salmonella* in meat, fruit, and other foods could lower the risk of contaminated food reaching the consumer. Use of a sensitive assay for detection of *Salmonella* in melons is also very important, since this food is eaten raw. The PCR assay evaluated in the current study could be used as a screening test, since results would be available in less time than with the cultural method. PCR-positive results could then be confirmed by the cultural method. Because the *invA* gene is present in pathogenic *Salmonella* serotypes, the PCR assay based on the primer pair targeting this gene could be applied for detection of *Salmonella* spp. that may be associated with particular food products, including poultry and food products that are consumed raw such as fruits and vegetables and/or ready-to-eat food. Further research will focus on validating the robustness of the *Salmonella* PCR assay in approved laboratories in Mexico for its use as a screening test using different types of samples, along with confirmation of the pathogen by the microbiological method. Additional studies to determine the most prevalent *Salmonella* serotypes found in beef and in cantaloupes and to determine the effect of environmental changes and the use of GAP on the prevalence of *Salmonella* in these foods are warranted.

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